Effects of the oral, direct factor Xa inhibitor apixaban on routine coagulation assays and anti-FXa assays

A. Hillarp, Kerstin Gustafsson, Lars Faxälv, K. Strandberg, F. Baghaei, I. Fagerberg Blixter, M. Berndtsson and Tomas Lindahl

Linköping University Post Print

N.B.: When citing this work, cite the original article.

Original Publication:
http://dx.doi.org/10.1111/jth.12649
Copyright: Wiley: 12 months
http://eu.wiley.com/WileyCDA/
Postprint available at: Linköping University Electronic Press
http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-111458
Effects of the oral, direct factor Xa inhibitor apixaban on routine coagulation assays and anti-Xa assays.

Andreas Hillarp*, Kerstin M. Gustafsson†, Lars Faxälv†, Karin Strandberg*, Fariba Baghaei‡, Inger Fagerberg Blixter‡, Maria Berndtsson§, Tomas L. Lindahl†.

From the *University and Regional Laboratories Region Skåne, Clinical Chemistry, Malmö, Sweden, †Department of Clinical and Experimental Medicine, Linköping University, Sweden the ‡Dept. of Medicine, Coagulation Centre, Sahlgrenska University Hospital, Gothenburg, Sweden and Institute of Biomedicine, Department of Clinical Chemistry and Transfusion Medicine, The Sahlgrenska Academy, University of Gothenburg, Sweden, and §Department of Clinical Chemistry, Karolinska University Hospital, Stockholm, Sweden.

Corresponding author: Tomas L Lindahl
Department of Clinical and Experimental Medicine
Linköping University
SE-581 83 Linköping, Sweden
Email: tomas.lindahl@liu.se

Running title
Effects of apixaban on coagulation assays.

Keywords
apixaban, direct Xa inhibitor, anticoagulants, coagulation assays, activated partial thromboplastin time, prothrombin time
Summary

Introduction: Apixaban is an oral direct factor Xa inhibitor developed for prophylaxis and treatment of thromboembolic disorders. Laboratory monitoring is not necessary, but the effects on common coagulation reagents and assays are clinically valuable information.

Objectives: To investigate the effect of apixaban on commonly used coagulation methods and evaluate anti-Xa assays for specific determination of the drug concentration.

Material and Methods: Apixaban was added to plasma from healthy subjects in the concentration range 0 -1,000 µg/L and analyzed using different reagents for activated partial thromboplastin time (APTT), prothrombin time (PT), antithrombin, protein C, and protein S. A lupus anticoagulant assay and an APTT with varying phospholipid concentrations were used to study the phospholipid dependence.

Results: In general, apixaban displayed fewer effects in vitro than what has been shown for rivaroxaban, another direct Xa inhibitor. The concentration needed to double the APTT varied between 2,200 and 4,700 µg/L and between 700 and 3,900 µg/L for PT reagents. The effect on antithrombin, protein C and S assays was dependent on the type of reagent. Apixaban did not cause false positive lupus anticoagulant results. Chromogenic anti-Xa assays displayed linear dose-response curves with apixaban.

Conclusions: Therapeutic concentrations of apixaban variably affect different assay groups, and even different reagents within an assay group. The effects were much smaller compared with rivaroxaban. Use of APTT and/or PT assays to screen the anticoagulant activity of apixaban cannot be recommended. Chromogenic anti-Xa assay can be deployed for reliable measurements of apixaban concentration.
Introduction
Therapeutic anticoagulation has been traditionally performed with a limited set of anticoagulant drugs, of which the vitamin K antagonists (VKA) and heparinoids (unfractionated heparin and low molecular weight heparin variants) are the most important. Many years’ experience of indirect inhibitors such as low molecular weight heparins and fondaparinux have shown factor Xa inhibition to be an effective anticoagulant strategy [1, 2]. However, these drugs have two important shortcomings; they require parenteral administration and are dependent on antithrombin, rendering them unable to inhibit factor Xa in the prothrombinase complex [3]. The first synthetic direct acting compound licensed for treatment of patients was rivaroxaban. Apixaban (Eliquis\textsuperscript{®}), from Bristol Myers Squibb (Princeton, NJ, USA), is one of the newest oral direct Xa inhibitors, with a molecular weight of 459.5, an inhibitor constant, $K_i$, of 0.08 nM for human Xa and with greater than 30,000-fold selectivity over thrombin and other coagulation enzymes [4]. Apixaban is currently approved for stroke prophylaxis in patients with non-valvular atrial fibrillation and as an alternative to heparins for thromboprophylaxis after hip and knee replacements [5]. Phase III clinical trials have recently shown an antithrombotic effect non-inferior to warfarin in acute venous thromboembolism and significantly fewer bleeding complications [6, 7]. The new oral anticoagulants (NOACs) have an advantage over VKAs in that the effect does not need to be monitored with laboratory analyses. However, as the drugs are, at least in part, excreted by the kidneys and/or metabolized by the liver, it may be necessary to assess the anticoagulant effect with a specific test to avoid drug accumulation in some patients [8]. Other clinical situations in which testing can be considered are in emergency situations such as major bleeds or preoperative preparation. According to the manufacturer of apixaban the effects are small and variable on common coagulation assays. Therefore, the prothrombin time (PT) and activated partial thromboplastine time (APTT) should not be used to evaluate the pharmacodynamic effect of the drug. However, there is also a need to know what general effects apixaban might have on PT and APTT assays as they are widely available and routinely performed laboratory tests. Another aspect is whether it is possible to conduct a thrombophilia investigation during apixaban treatment. There are only limited reports on the pharmacokinetic and pharmacodynamic profiles of apixaban [7-10], including one, more extensive in vitro study of the effects on routine and more specialized coagulation assays [9] and one
very recent study that investigated the effects of various APTT and PT reagents together with an evaluation of three different anti-Xa assays [10]. Both studies utilized pooled normal plasma spiked with apixaban. In the current study, we have used plasma samples from ten healthy individuals spiked with the pure apixaban substance to investigate the anticoagulant activities over a wide concentration. The effects on common routine coagulation and analyses in the thrombophilia investigation have been studied as has the inter-individual variation in the effect.
Material and Methods

Study design

A working group, representing the coagulation laboratories at four Swedish university hospitals (Gothenburg, Linköping, Malmö and Stockholm), undertook the task to investigate the effects of apixaban on common coagulation assays. The study design involved a similar approach as the same working group used to investigate the in vitro effects of dabigatran [11] and rivaroxaban [12]. In brief, plasma samples from 10 different healthy individuals were spiked with apixaban at 10 different concentrations and aliquots were investigated at the four participating laboratories. All assays were divided between the participants, i.e. one lab investigated the effects on the different APTT reagents and another lab ran all the PT assays etc. As the preliminary results indicated that apixaban had quite different in vitro effects compared to the previously investigated drug rivaroxaban additional tests and direct comparisons between the drugs were performed. These included lupus anticoagulant assay and a home-made APTT with varying phospholipid concentrations. The selected plasma concentration interval was based on the expected concentrations obtained from published dose-ranging studies [7, 13,14]. In the APPRAISE-1 study [15] on treatment of acute coronary syndrome, 2.5 mg and 10 mg twice-daily were used and resulted in a peak concentration of 68 (38/98; 5th/95th percentile) and 267 μg/L (122/412 μg/L; 5th/95th percentile), respectively [7]. The corresponding trough concentrations were 57 (18/97; 5th/95th percentile) and 221 µg/L (30/412; 5th/95th percentile), respectively. A twice-daily dose of 5 mg apixaban has been proposed as therapeutic strategy for stroke prophylaxis in patients with non-valvular atrial fibrillation. Plasma concentration profiles during different apixaban dosage regimens has been calculated from the results of a phase III trial [16], and suggested that 5 mg twice-daily would result in a median trough concentration of 107 μg/L (56/203 μg/L; 10th/90th percentile). Based on these data and to facilitate comparison with our previous report on rivaroxaban [12], we decided to highlight our results from the APTT and PT analyses with a maximum concentration of 200 μg/L and a trough concentration at 50 μg/L. In our study, we included up to 5-fold higher concentrations (1,000 μg/L) than the expected peak level, which would simulate a result of drug accumulation.
Materials

Apixaban was provided from the manufacturer, Bristol Myers Squibb; Rivaroxaban was provided from the manufacturer Bayer Schering Pharma AG. Stock preparations were made by dissolving the drugs in 100 % dimethyl sulphoxide (DMSO, Merck, Darmstadt, Germany). The evacuated blood collection tube system was from BD (Franklin Lakes, NJ, US). A phospholipid mixture, used for a local APTT method, containing phosphatidyl choline (42 %), phosphatidyl serine (28 %) and sphingomyelin (30 %) was purchased from Rossix AB (Mölndal, Sweden).

Production of plasma samples and apixaban calibrator

Human blood from 10 adult healthy volunteers, both men and women (five of each gender), was collected in evacuated plastic tubes containing 1:10 volume of 0.109 mol/L tri-sodium citrate as anticoagulant. Ten tubes, in total 50 mL citrated blood, were obtained from each individual. The tubes were centrifuged at 2,000 x g for 20 min, and the platelet-poor plasma from each individual was pooled and stored frozen at -70°C. The plasma was thawed in a 37 °C water bath for 15 min, divided into 20 aliquots of 1.0 mL each, “spiked” with apixaban to final concentrations ranging from 0.0 to 1,000 µg/L and then stored at -70°C until they were transported on dry ice to the participating laboratories for analysis.
Pooled normal plasma (n=45 healthy donors) was used to produce a calibrator set, containing 0, 25, 50, 100, 200 and 400 µg/L apixaban, for the anti-Xa assays that was used to quantitate the amount of apixaban in the individual plasma samples. Pooled plasma, spiked with apixaban or rivaroxaban, was also used to evaluate the effects on additional tests involving lupus anticoagulant and an APTT assay with varying phospholipid content. All samples and calibrators were prepared so that the final DMSO concentration of became 1 % (v/v), irrespective of the drug concentration.

Routine coagulation analyses

The activated partial thromboplastin time (APTT) was assayed using 5 different reagents; Actin FSL from Siemens Healthcare Diagnostics (Deerfield, IL, US); PTT-Automate from Stago; APTT-SP IL test liquid from Instrumentation Laboratory SpA (Milano, Italy); TriniCLOT aPTT HS from Trinity Biotech (Bray, Ireland); and APTT-
DG from Grifols (Barcelona, Spain). The APTT assays were performed on a BCS-XP instrument from Siemens Healthcare Diagnostics according to the manufacturers’ instructions, i.e., equal volumes of sample and APTT reagent are mixed and incubated (3 or 5 minutes depending on reagent) at 37°C and then one volume of 25 mM calcium chloride solution is added to the mixture and the clotting time determined.

The prothrombin time (PT) was performed with both plain (Quick type) and combined (Owren type) PT reagents. Five common Quick type PT assays were tested: Dade Innovin and Thromborel S (Siemens Healthcare Diagnostics); STA-Neoplastine CI Plus (Stago); RecombiPlastTin 2G (Instrumentation Laboratory); and Technoplastin HIS (Technoclone GmbH, Vienna, Austria). Three different Owren type PT assays were also investigated: Stago prothrombin complex assay, SPA+, from (Stago); Nycotest PT from Axis-Shield plc (Dundee, Scotland); and PT Owren from MediRox AB (Nyköping, Sweden). The Quick PT assays were calibrated using local determination of ISI and mean normal PT for each reagent. The Owren PT assays were all calibrated using the national INR calibrators certified by the Swedish external quality assessment organization EQUALIS (Uppsala, Sweden) traceable to a WHO reference thromboplastin [17]. The PT assays were performed on an ACLTop (Instrumentation Laboratory SpA) coagulation instrument according to standard procedures.

Two chromogenic reagents for antithrombin activity were evaluated; Innovance Antithrombin, based on Xa inhibition from Siemens Healthcare Diagnostics and Stachrom ATIII from Stago that utilizes thrombin inhibition. The Innovance Antithrombin assay was performed on the Sysmex CS2100i instrument from Siemens Healthcare Diagnostics, whereas the Stachrom ATIII test was run on the STA-R Evolution analyzer from Stago according to the manufacturer’s instructions.

Protein C was measured with one chromogenic method, Coamatic Protein C from Chromogenix (Milano, Italy) and two clot-based assays: Protein C coag (Siemens Healthcare Diagnostics) and Staclot Protein C (Stago). The Protein C coag and the Coamatic Protein C assays were performed on the BCS-XP instrument whereas the
Staclot Protein C was run on the STA-R Evolution analyzer according to the manufacturer's instructions.

Assays to assess protein S in plasma involved Coamatic Protein S free (Chromogenix), which measures free protein S antigen and two clot-based assays: Protein S Ac (Siemens) and Staclot Protein S (Stago). The Coamatic Protein S free and Protein S Ac assays were performed on the BCS-XP instrument, whereas the Staclot Protein S was run on the STA-R Evolution analyzer according to the manufacturer's instructions.

*Anti-Xa assays used for quantitation of apixaban in plasma.*

At the time of the study there were no commercial available assays or calibrators for apixaban measurements and the participating laboratories used their local anti-Xa method in clinical use for measuring rivaroxaban. The STA Liquid anti-Xa assay from Stago was used by two laboratories, in one case on the Sysmex CS2100i instrument and in the other on STA-R Evolution. The other two laboratories used the Coamatic Heparin reagent from Chromogenix on BCS-XP and ACLTop, respectively. Both assays are without addition of exogenous antithrombin, which otherwise could compromise the results [18]. A common calibrator set was used for all participants and was based on pooled normal plasma spiked with apixaban in concentrations between 0 – 400 µg/L. Samples that exceeded the highest calibration concentration of 400 µg/L were diluted with pooled normal plasma and re-analyzed, and the results were multiplied by the dilution factor (1:2 or 1:4).

*Other assays used for direct comparison between apixaban and rivaroxaban*

The LA1 screening reagent and LA2 confirmation reagent (Siemens) were used as an integrated dilute Russell's viper venom time (dRVVT) test to evaluate and compare the effect of anti-Xa drugs rivaroxaban and apixaban. The test was performed on the BCS-XP instrument, and the result is expressed as an LA1/LA2 ratio. According to the manufacturer, a ratio between 1.2 and 1.5 is considered weakly positive or border line, a ratio between 1.5 and 2.0 is classified as moderately positive, and a ratio above 2.0 is strongly positive.
The inhibitory effect of apixaban and rivaroxaban at different phospholipid concentrations was measured with a local APTT method in an Amelung KC4 instrument (Sigma, Lemgo, Germany). The assay utilized SiO₂ (final conc. 17 mg/mL) as activator and varying concentrations of a phospholipid mixture. Samples of pooled normal plasma with or without inhibitors, (apixaban or rivaroxaban at 400 µg/L) were mixed with of activator and 0, 1.7, 17, 26, 35, 43 or 52 µM phospholipids and after recalcification with 18 mM CaCl₂ the coagulation time was determined.

Statistics
Results are presented as mean ± standard deviation (SD). Concentrations required to double the PT or APTT (CT₂) and linear regression equations for anti-Xa activity assays were calculated using Excel 97 software (Microsoft®). For PT assays the CT₂ values were calculated by linear regression analysis, and for the APTT assays a 2nd order polynomial function was chosen.
Results
The effects of increasing apixaban concentrations on the five different APTT reagents were similar non-linear and flat dose-response curves (Fig 1). Assuming a peak concentration of 200 µg/L, the responses were within the reference interval of each reagent group for most samples. Even at the highest concentration of 1,000 µg/L, the APTT remained below the upper reference limit for several samples in each APTT reagent group. The effect on the APTT, expressed as the concentration required doubling the APTT (CT₂), was calculated to be between 2,203 and 4,666 µg/L for the five different reagents (Table 1), which is a relative 2.1-fold difference. The inter-individual variability is reflected to the same extent over the entire measuring range, i.e. the individual dose-response curves did not cross each other, and the same plasma donor that expressed the shortest or the longest APTT did so for each apixaban concentration (not shown).

There was a linear dose-response effect on the nine different PT assays with a wide variation. There was, however, no clear difference in sensitivities for apixaban between Quick PT and Owren PT type reagents (Fig. 2). The least sensitive reagent was the Dade Innovin, with a mean increase of 0.02 INR per 100 µg/L calculated for the concentration range 0 – 1,000 µg/L; the calculated CT₂ was almost 4,000 µg/L (Table 2). None of the samples from the 10 donors had an increased INR above the upper reference range (>1.2) at an expected therapeutic peak concentration of 200 µg/L. Two of the Owren PT type reagents had a response very similar to Dade Innovin with minute effects on the INR at therapeutic apixaban concentrations. The most sensitive reagent was Recombiplastin followed by Technoplastin and Neoplastine CI plus, all three belonging to the Quick PT type reagents. The mean increase of INR per 100 µg/L apixaban was 0.15, which means that the majority of donors were just above the upper reference range of the assay at an expected peak concentration. By comparing the CT₂ values the relative difference between the eight different PT assays was 5.6-fold (Table 2).

The antithrombin activity assay based on factor Xa inhibition (Innovance Antithrombin) increased with the apixaban concentration whereas the other assay (Stachrom ATIII), based on thrombin inhibition, were not affected (not shown).
Already at expected trough levels of apixaban, around 50-100 µg/L, the factor Xa-based antithrombin level reached the upper measuring range of the assay. In some of the samples with a concentration of 200 µg/L and in all samples with higher concentrations, no quantitative value could be obtained.

Protein C levels measured with coagulation-based assays were affected to some extent by apixaban, whereas the chromogenic assays were not (fig. 3). The Staclot Protein C assay from Stago was more sensitive than the Siemens Protein C coag assay meaning that the Staclot protein C levels were overestimated to a greater extent compared to the Siemens assay. Expressed as an increase in the protein C activity per 100 µg/l of the drug, assuming a linear dose-response model, the Staclot Protein C displayed an incremental increase of 0.04 IU/ml per 100 µg/l apixaban. The Protein C coag assay was not affected at apixaban concentrations below 100 µg/L; at higher concentrations, the average increase was less than 0.02 IU/ml per 100 µg/L of the drug.

Coagulation-based assays for protein S appeared to be more affected than the coagulation-based protein C assays. At peak concentrations of apixaban, 200 µg/L, all samples were above the upper measuring range using the Protein S Ac kit from Siemens; with the Staclot Protein S from Stago, 5 of 10 samples were above the measuring range given for this particular assay (not shown). Based on the obtained measurements in the low concentration of the drug, 0 – 100 µg/L, the Protein S Ac assay increased by an average of 0.22 IU/ml per 100 µg/L apixaban compared with the corresponding value of 0.13 IU/ml per 100 µg/L apixaban for Staclot protein S assay. The free protein S antigen results, obtained with the Coamatic Protein S free kit, were slightly reduced as apixaban increased, with a mean reduction of 11 % at the highest apixaban concentration. At a therapeutic concentration (200 µg/L), the change in free protein S antigen among the 10 different donor plasmas ranged from -10 to +3 %, with a mean reduction of 3.9 %.

The apixaban concentration in the spiked plasma samples was determined with two different anti-Xa assays, each performed on two different analytical instruments. The linear correlation between the expected concentration and the measured concentration is shown in Figure 4. The calculated recoveries were between 88%
and 119 % of the expected values for all eight concentrations between 25 and 1,000 µg/L (table 3). At the very low measuring range (10 µg/L), one assay protocol resulted in an overestimation of almost 100 %.

The effects on an integrated dRVVT test, manufactured for detection of lupus anticoagulant, were evaluated with increasing concentrations of rivaroxaban or apixaban in pooled normal plasma. It was shown that rivaroxaban affected the LA1 reagent, with low phospholipid, to a greater extent than apixaban did, whereas with the phospholipid-rich LA2 reagent, the dose-response curves were similar for both Xa inhibitors (fig. 5). This gave (false) positive lupus anticoagulant ratios at rivaroxaban concentrations of 100 µg/L and higher, whereas the lowest apixaban concentration that resulted in a weak positive ratio was 600 µg/L (not shown).
Discussion
Apixaban is an oral, direct FXa inhibitor that not only has the capacity to inactivate free FXa but also clot-associated FXa activity and the FXa that is part of the prothrombinase complex [19]. Thus, apixaban has the potential to down-regulate the coagulation processes very efficiently and therefore may interfere in many coagulation assays in which FXa activity has an impact on the results obtained.

We observed that APTT and PT assays were weakly sensitive in general, but that there were differences between assays, which have also been acknowledged by the manufacturer. APTT was within the reference range for most donor samples at 200 µg/L, and PT was not above reference range or only slightly above, in accordance with previous studies on samples from patients with acute coronary syndrome [7] and healthy volunteers [12]. The recent in vitro studies by Douxfils et al. [9] and Gouin-Thibault et al. [10] showed similar low sensitivities with other reagents for PT and APTT, indicating that the relatively weak apixaban effect is generalizable to many screening assays of the coagulation system. Thus, a normal APTT or a normal PT does not exclude a peak concentration of apixaban and is of no use in estimating the concentration of apixaban. A strength of our study is that it show the inter-individual variation for the routine coagulation assays. The broad range in APTT for the 10 healthy individual at peak or 5 times peak concentration (Table 1) illustrates the difficulties in evaluating an individual APTT result during apixaban therapy. Another observation is that the concentrations needed to double APTT (CT₂) were for most reagents a factor of 5-6 times higher than for rivaroxaban [12]. However, for one of the reagents, Actin FSL, the CT₂-value was 11 times higher than for rivaroxaban [12].

Apixaban had weak effects on the tested PT assays. At 200 µg/L only two reagents resulted in a mean INR that was above the upper reference limit of 1.2. Even at the highest apixaban concentration some samples were still in the normal range. We grouped the PT assays in Quick and Owren types as it was assumed that the Quick PT assays would be more sensitive compared to the Owren PT, which has previously been shown with the oral anticoagulants dabigatran [11] and rivaroxaban [12]. The basis for this assumption is the differences in plasma dilution that is 1:3 for Quick PT compared to 1:21 for the Owren PT and this renders the Owren PT assay being less susceptible to various interfering substances including lupus anticoagulant. However,
we found that the least sensitive PT reagent was the Quick type Innovin. The reason for this insusceptibility is unknown. Two recent in vitro studies resulted in similar findings that Innovin was the least sensitive PT reagent [9, 10]. All PT assays were affected to a lesser extent by apixaban in comparison with rivaroxaban. The relative sensitivity, illustrated by the CT2 values, revealed that 2 – 7 times higher concentrations of apixaban were needed to double the PT compared to rivaroxaban. In line with this observation is a study performed with rabbits that showed that rivaroxaban prolonged the APTT and PT more than apixaban [20].

The effect on the dRVVT test was also less than expected given the fact that rivaroxaban can result in false positive lupus anticoagulant results [21 - 23]. The dose-response curves in Figure 5 indicated that rivaroxaban and apixaban differed more in the LA1 screening test with limited phospholipid compared with the LA2 reagent that contained more phospholipid. Our observation is made with a single LA-reagent and the results must be interpreted with caution but similar dose-dependent and equal effects on the screen and confirm tests, with low and high phospholipid content, has been reported for apixaban with two other lupus anticoagulant assays [9]. Thus, under therapeutic concentrations with apixaban, it may be possible that the dRVVT test will not give the false positive results shown with rivaroxaban. The reason for this discrepancy might be different sensitivities to the phospholipid content in the reagents. This was supported by our experiment based on the APTT with varying concentrations of added phospholipids that showed that the clotting time in samples containing apixaban were unaffected whereas coagulation times were dependent on the phospholipid contents in samples with rivaroxaban.

There is no obvious explanation for the differences between these two direct factor Xa inhibitors. As the molecular weight is similar – 459.5 Da for apixaban [4] and 436 Da for rivaroxaban [24] – the molar concentration is very similar at the same mass concentration. The inhibitor constant, Ki, which is an indicator how potent an inhibitor is, for human factor Xa is lower for apixaban than for rivaroxaban, 0.08 nM and f 0.4 nM, respectively. This means that apixaban is slightly more potent in inhibiting Xa compared to rivaroxaban and cannot explain the observed differences in vitro. Both drugs have a >10,000-fold greater selectivity for factor Xa than for other serine proteases [4, 24]. Thus the difference in effects on APTT and PT between
rivaroxaban and apixaban remains to be explained although our results suggest that the phospholipids in the investigated reagents seem to play a role. We would like to stress that this is an in vitro observation only, and there is no relation between these kind of effects and clinical effects for any of the new oral anticoagulants.

Chromogenic anti-Xa assays are the standard methodology for measuring the concentrations of indirect and direct Xa inhibitors. Two different assays were used on two different instruments for each reagent type with a programming recommended by the manufacturer for measuring the direct Xa inhibitor rivaroxaban. In the absence of a commercially available calibrator, the participating laboratories used identical sets of apixaban calibrators that were prepared by spiking the drug into pooled normal plasma. There were linear relationships between the expected and observed anti-Xa activities with good correlation for all combinations. Furthermore, equivalence expressed as the average recovery in percentage, was close to 100 % (range 95 – 103 %), indicating that the anti-Xa assays used are suited to measuring apixaban when a drug-specific calibrator is used.

In conclusion, the APTT and PT are not useful for measuring the plasma concentration of apixaban, not even to obtain a crude estimate, unless the locally used assays are tested and validated for the effect. If the aim is to measure the pharmacodynamic effect of apixaban, more specific assays, preferably based on factor Xa inhibition, should be used. Furthermore, a useful laboratory screening for thrombophilia cannot be performed during apixaban therapy without knowledge of the influence on the locally used assays. The results obtained in our study will facilitate for laboratories to adequately estimate the effect of the drug on common coagulation assays.
Addendum
All authors contributed to the design of the study, interpretation of data and final approval of manuscript. A Hillarp and TL Lindahl wrote the manuscript. KM Gustafsson drew the figures. K Strandberg, F Baghei and KM Gustafsson contributed to the revision of the manuscript. KM Gustafsson performed experiments on PT and anti-Xa, I Fagerberg Blixter and M Berndtsson on protein C, protein S, antithrombin and anti-Xa and L Faxälv on effects of phospholipids on APTT.

Acknowledgements
The expert technical assistance of the following biomedical scientists is gratefully acknowledged: Karin Erlin (Linköping) Margareta Persson (Malmö); Lisbeth Söderblom and Eva-Marie Norberg (Stockholm); Ewa Lönn-Karlsson (Linköping); Susanne Samuelsson and Barbro Christenson (Gothenburg). We are also thankful for all support from Mrs Elisabeth Nilsson and Dr Gunnar Nordin at the EQUALIS office in Uppsala. The rivaroxaban was a kind gift from Bayer AG. The apixaban used in this study was a kind gift from Bristol Meyers Squibb, who also provided partial funding of the study.
References


Martinuzzo ME, Barrera LH, D’adamo MA, Otaso JC, Gimenez MI, Oyhamburu J. Frequent False-positive results of lupus anticoagulant tests in plasmas of patients receiving the new oral anticoagulants and enoxaparin. Int J Lab Hematol 2014; 36: 144-150.

Table 1. Mean APTT (seconds) at different concentrations of apixaban and concentrations required to double the APTT (CT2).

<table>
<thead>
<tr>
<th>Type of APTT</th>
<th>Baseline (0 µg/L) Mean (±SD) (range)</th>
<th>“Trough” (50µg/L) Mean (±SD) (range)</th>
<th>“Peak” (200 µg/L) Mean (±SD) (range)</th>
<th>“5x overload” (1000 µg/L) Mean (±SD) (range)</th>
<th>Mean CT2 (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin FSL</td>
<td>27.2 (±2.3) (23.2-30.5)</td>
<td>29.1 (±2.6) (24.6-32.5)</td>
<td>31.3 (±2.9) (25.9-35.2)</td>
<td>38.5 (±4.1) (31.2-44.4)</td>
<td>4666</td>
</tr>
<tr>
<td>TriniClot</td>
<td>29.6 (±3.7) (25.3-35.2)</td>
<td>31.4 (±4.4) (26.3-37.5)</td>
<td>34.5 (±5.1) (28.4-42.1)</td>
<td>43.1 (±7.7) (35.0-55.7)</td>
<td>3855</td>
</tr>
<tr>
<td>APTT-SP</td>
<td>30.9 (±3.8) (26.0-36.2)</td>
<td>33.8 (±4.4) (27.7-39.9)</td>
<td>36.8 (±5.2) (30.4-44.4)</td>
<td>48.1 (±8.3) (36.6-60.9)</td>
<td>2723</td>
</tr>
<tr>
<td>PTT-A</td>
<td>30.8 (±3.3) (25.8-36.7)</td>
<td>33.0 (±4.2) (27.4-40.7)</td>
<td>36.7 (±5.0) (29.3-46.0)</td>
<td>47.7 (±9.2) (37.1-66.9)</td>
<td>2727</td>
</tr>
<tr>
<td>APTT-DG</td>
<td>25.2 (±3.5) (20.0-32.9)</td>
<td>28.1 (±4.1) (22.1-36.7)</td>
<td>31.6 (±4.6) (24.6-41.0)</td>
<td>41.5 (±7.6) (31.3-57.3)</td>
<td>2203</td>
</tr>
</tbody>
</table>
Table 2. Mean INR at different concentrations of apixaban, mean INR increase per 100 µg/L and concentrations required to double the prothrombin time (CT₂).

<table>
<thead>
<tr>
<th>PT-assay</th>
<th>“Trough” (50µg/L) Mean (±SD)</th>
<th>“Peak” (200 µg/L) Mean (±SD)</th>
<th>“5x overload” (1000 µg/L) Mean (±SD)</th>
<th>Mean increase of INR/100 µg/L</th>
<th>CT₂ Mean µg/L (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quick type PT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thromborel S</td>
<td>0.93 (±0.04)</td>
<td>1.03 (±0.08)</td>
<td>1.53 (±0.17)</td>
<td>0.06</td>
<td>1876 ±193</td>
</tr>
<tr>
<td>Dade Innovin</td>
<td>0.95 (±0.05)</td>
<td>0.99 (±0.05)</td>
<td>1.14 (±0.08)</td>
<td>0.02</td>
<td>3911 ±280</td>
</tr>
<tr>
<td>Technoplatin</td>
<td>1.03 (±0.07)</td>
<td>1.24 (±0.11)</td>
<td>2.47 (±0.39)</td>
<td>0.15</td>
<td>919 ±121</td>
</tr>
<tr>
<td>Neoplastine</td>
<td>1.00 (±0.07)</td>
<td>1.18 (±0.09)</td>
<td>2.23 (±0.32)</td>
<td>0.13</td>
<td>1254 ±126</td>
</tr>
<tr>
<td>Recombiplastin</td>
<td>1.06 (±0.06)</td>
<td>1.29 (±0.08)</td>
<td>2.25 (±0.24)</td>
<td>0.13</td>
<td>704 ±59</td>
</tr>
<tr>
<td>Owren type PT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPA+</td>
<td>1.05 (±0.06)</td>
<td>1.09 (±0.07)</td>
<td>1.29 (±0.09)</td>
<td>0.03</td>
<td>3091 ±278</td>
</tr>
<tr>
<td>Nycotest PT</td>
<td>1.02 (±0.06)</td>
<td>1.08 (±0.07)</td>
<td>1.30 (±0.12)</td>
<td>0.03</td>
<td>3023 ±283</td>
</tr>
<tr>
<td>Owren’s PT</td>
<td>1.03 (±0.07)</td>
<td>1.14 (±0.08)</td>
<td>1.59 (±0.15)</td>
<td>0.06</td>
<td>1889 ±112</td>
</tr>
</tbody>
</table>
Table 3. Determination of apixaban with chromogenic anti-Xa assays.

<table>
<thead>
<tr>
<th>Type of assay/instrument</th>
<th>Linear regression&lt;sup&gt;1&lt;/sup&gt; (y = kx + I)</th>
<th>Correlation coefficient&lt;sup&gt;1&lt;/sup&gt; (r²)</th>
<th>Average recovery&lt;sup&gt;2&lt;/sup&gt; % ±SD (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coamatic + ACLTop</td>
<td>1.05x - 7.2</td>
<td>0.997</td>
<td>95 ±6 (91 – 107)</td>
</tr>
<tr>
<td>Coamatic + BCS-XP</td>
<td>1.00x - 0.8</td>
<td>0.993</td>
<td>103 ±7 (92 – 116)</td>
</tr>
<tr>
<td>Liquid Xa + STA-R</td>
<td>0.87 + 14.7</td>
<td>0.997</td>
<td>96 ±10 (88 – 119)</td>
</tr>
<tr>
<td>Evolution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liquid Xa + Sysmex CS2100i</td>
<td>1.00 + 5.5</td>
<td>0.998</td>
<td>102 ±5 (91 – 107)</td>
</tr>
</tbody>
</table>

1) The linear regression and correlation coefficient is calculated with the expected apixaban concentration on the x axis and the measured concentrations on the y axis (see also Fig. 4).

2) Recovery is calculated as the (measured value/expected value *100), and the reported average is the mean for all eight concentrations between 25 and 1,000 µg/L.
**Figure 1.** Effect of apixaban on the APTT. The obtained APTTs using five different reagents were plotted against the apixaban concentration in plasma. Triniclot aPTT HS (○), Actin FSL (○), PTT-Automate (△), APTT-SP (▼) and DG-APTT (■). Results are shown as seconds (mean + SD) of 9 different healthy donors.
**Figure 2.** Effect of apixaban on the PT assay expressed as INR. A) Analysis of five Quick PT assays based on plain thromboplastin reagents: STA-Neoplastine (●), RecombiPlastTin 2G (○), Technoplatin HIS (▼), Thromborel S (△), and Dade Innovin (■). B) Analysis of three Owren type PT assays, based on rabbit thromboplastin reagents: SPA+ (○), Nycotest PT (▼), and Owren’s PT (○). Results are shown as INR (mean + SD) of 10 different healthy donors.

**Figure 3.** Effect of apixaban on Protein C assays. Two coagulation-based assays, Protein C coag (●) and Staclot Protein C (▼), and two chromogenic assays, Coamatic Protein C (△) and Berichrom Protein C (○), were used in the evaluation. Results are shown as the activity (IU/mL; mean + SD) of 9 different healthy donors.
Figure 4. Determination of the apixaban plasma concentration by anti-Xa assays. Two assays, Liquid anti-Xa and Coamatic Heparin, were evaluated, and each assay was analyzed on two different instruments with the following combinations: Coamatic Heparin reagent on ACLTop (▼) or BCS-XP (●) and Liquid Xa on STA-R Evolution (○) and Sysmex CS2100i (▲). The results are shown as the calculated concentration in µg/L (mean + SD) of 10 healthy donors.
Figure 5. Effects of direct anti-Xa inhibitors on the lupus anticoagulant test. Samples of pooled normal plasma were spiked with rivaroxaban (○) or apixaban (●) in 10 different concentrations ranging from 0 µg/L to 1,000 µg/L and analyzed with an integrated dRVVT assay. The assay consists of an LA1 screening (low phospholipid) and an LA2 confirmation (phospholipid rich) clotting test. The results are shown as the ratio LA1/LA2 test. The hatched lines indicate the limits for negative, borderline and positive lupus anticoagulant test results.